microRNA-192 suppresses the expression of the farnesoid X receptor

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Abstract: Farnesoid X receptor (FXR, NR1H4) plays an important role in the regulation of bile acid homeostasis in liver and intestine and may exert protective effects against certain forms of cancer such as colon carcinoma. However, the role of FXR in cell growth regulation, apoptosis, and carcinogenesis is still controversial. Similar to FXR, microRNA-192 (miR-192) is mainly expressed in the liver and colon and plays an important role in the pathogenesis of colon carcinoma. In this study, we investigated the extent to which FXR is regulated by miR-192. Two in silico-predicted binding sites for miR-192-3p within the NR1H4-3' untranslated region (UTR) were examined in vitro by luciferase reporter assays. Wild-type and mutated forms of the NR1H4-3'UTR were subcloned into a pmirGLO vector and cotransfected into Huh-7 cells with miR-192-3p. To study the effects of miR-192 on the expression of FXR, FXR target genes and cell proliferation, Huh-7 and Caco-2 cells were transfected with miR-192-5p and -3p mimics or antagomirs. In addition, the correlation between FXR and miR-192 expression was studied by linear regression analyses in colonic adenocarcinoma tissue from 27 patients. MiR-192-3p bound specifically to the NR1H4-3'UTR and significantly decreased luciferase activity. Transfection with miR-192 led to significant decreases in NR1H4 mRNA and protein levels as well as the mRNA levels of the FXR-inducible bile acid transporters OST -OST and OATP1B3. Significant inverse correlations were detected in colonic adenocarcinoma between NR1H4 mRNA and miR-192-3p expression. In summary, microRNA-192 suppresses the expression of FXR and FXR target genes in vitro and in vivo.

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microRNA-192 suppresses the expression of the farnesoid X receptor

Abbreviated title: miR-192 suppresses the expression of FXR

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Contributorship

Participated in research design: Krattinger, Mwinyi, Kullak-Ublick; Conducted experiments: Krattinger; Data acquisition and analysis: Krattinger, Boström, Thasler; Wrote or contributed to the writing of the manuscript: Krattinger, Mwinyi, Kullak-Ublick, Schiöth.

ABSTRACT

Farnesoid X receptor (FXR, *NR1H4*) plays an important role in the regulation of bile acid homeostasis in liver and intestine, and may exert protective effects against certain forms of cancer such as colon carcinoma. However, the role of FXR in cell growth regulation, apoptosis and carcinogenesis is still controversial. Similar to FXR, microRNA-192 (miR-192) is mainly expressed in the liver and colon, and plays an important role in the pathogenesis of colon carcinoma. In this study, we investigated the extent to which FXR is regulated by miR-192. Two *in silico*-predicted binding sites for miR-192-3p within the *NR1H4*-3′ untranslated region (UTR) were examined *in vitro* by luciferase reporter assays. Wild-type and mutated forms of the *NR1H4*-3′UTR were subcloned into a pmirGlo vector and co-transfected into Huh-7 cells with miR-192-3p. To study the effects of miR-192 on the expression of FXR, FXR target genes and cell proliferation, Huh-7 and Caco-2 cells were transfected with miR-192-5p and -3p mimics or antagomirs. In addition, the correlation between FXR and miR-192 expression was studied by linear regression analyses in colonic adenocarcinoma tissue from 27 patients. MiR-192-3p bound specifically to the *NR1H4*-3′UTR and significantly decreased luciferase activity. Transfection with miR-192 led to significant decreases in *NR1H4* mRNA and protein levels as well as the mRNA levels of the FXR-inducible bile acid transporters OSTα-OSTβ and OATP1B3. Significant inverse correlations were detected in colonic adenocarcinoma between *NR1H4* mRNA and miR-192-3p expression. In summary, microRNA-192 suppresses the expression of FXR and FXR target genes *in vitro* and *in vivo*.

KEYWORDS

miR-192 - farnesoid X receptor – bile-acid transporters - drug-induced liver injury – colonic adenocarcinoma

INTRODUCTION

Nuclear farnesoid X receptor (FXR, *NR1H4*) is a ligand-activated transcription factor that plays a crucial role in the regulation of bile acid, cholesterol, lipid and glucose homeostasis. It is mainly expressed in the liver, intestines, kidney, and adrenal glands [34]. FXR regulates key genes involved in human bile acid synthesis and metabolism, including bile acid transporters [4]. Studies on FXR knockout mice have shown that FXR exerts hepatoprotective effects. Diminished FXR expression has been linked to an increase in inflammatory responses and neoplastic transformation in mice [7,16]. Mice lacking FXR expression show elevations in serum and hepatic bile acid levels and a higher incidence of hepato(cholangio)cellular carcinoma [12,33]. In mouse intestine, loss of FXR and subsequent elevations of intestinal bile acid concentrations lead to earlier mortality caused by increased tumor progression via promotion of Wnt signaling. FXR may play a key role in the intestinal defense against potentially toxic bile acids by regulating their transport, detoxification, and neosynthesis [8,23]. Decreased FXR expression levels in human colon cancer tissue compared with non-neoplastic tissue are associated with adverse clinical outcome [15]. In contrast, strongly enhanced FXR expression, leading to altered expression of FXR-regulated drug uptake transporters, confers chemoresistance in cancer patients [22]. The role of FXR in cell growth regulation, apoptosis and carcinogenesis is controversially discussed in the literature. An immunohistochemical study, for example, showed preserved or enhanced FXR protein expression in tumor cell nuclei of human hepatocellular carcinoma tissue compared with hepatocyte nuclei of normal and diseased liver [14].

MicroRNAs (miRs) are short non-coding RNA molecules of 18–25 nucleotides in length, which repress specific target mRNAs by degradation or translational repression [2]. Two important liver-specific miRNAs are miR-122, which is estimated to comprise 70% of the total hepatic miR pool in adults, and miR-192 [28]. The gene encoding hsa-miR-192 is

50 located on chromosome 11. In addition to expression in the liver, miR-192 is found in the
51 kidneys and gastrointestinal tract [20]. Elevated serum levels of miR-192 have been detected
52 in various liver-associated diseases, including drug-induced liver injury, non-alcoholic
53 steatohepatitis, cholangiocarcinoma, and hepatitis-B-related hepatocellular carcinoma, and
54 may serve as a biomarker [24,26,28,31,37]. Thus, several studies have hypothesized that
55 tissue-specific chronic inflammation may trigger increases in miR-192 expression, as in non-
56 alcoholic steatohepatitis [21,24,26]. Because miR-192 shows an inverse correlation with the
57 metastatic potential of colon cancer cells, miR-192 has been suggested to be a predictive
58 biomarker for the risk of developing liver metastasis in colon carcinoma. *In vivo* studies in
59 mice suggest that miR-192 targets B-cell lymphoma 2 (*BCL2*), zinc-finger E-box-binding
60 homeobox 2 (*ZEB2*), and vascular endothelial growth factor A (*VEGFA*), all of which are
61 important anti-apoptotic and angiogenic regulators [9]. The tumor suppressor protein p53 can
62 act as a transcription factor within the miR-192 promoter, whereas miR-192 itself appears to
63 suppress carcinogenesis by promoting p21 accumulation [3,27]. Loss of p53 functions by
64 mutation and consequently decreased expression of miR-192 has been suggested to be a key
65 step in colon carcinogenesis [3,11,25,29]. Although, miR-192 is not considered to be a
66 typically dysregulated microRNA in several studies on hepatocellular carcinoma (HCC), Lian
67 et al. showed a significantly suppressed expression of miR-192 in HCC tissue as compared to
68 non-tumorous tissue [19]. An important role of miR-192 targeting *ZEB2* mRNA has also been
69 found in HCC [13]. In contrast, certain forms of cancer, such as cholangiocarcinoma and
70 esophageal cancer, show increased miR-192 expression during carcinogenesis [21,26].

71 In this study, we investigated to what extent miR-192 modulates the expression of FXR and
72 thereby affects the expression of FXR target genes in liver and colon cancer-derived cell lines.

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MATERIAL AND METHODS

Bioinformatics. An *in silico* search for possible miRNA-binding sites in the 3′ untranslated region (UTR) of the *NR1H4* gene was performed using miRANDA (Memorial Sloan-Kettering Cancer Center, New York, NY, USA), DIANA-microT-CDS (Biomedical Science Research Center Alexander Fleming, Athens, Greece), and miRBase (Faculty of Life Science, University of Manchester, Manchester, UK). mRNA and miR expression data from 27 colonic adenocarcinoma tissue samples (E-GEOD-29623, Affymetrix GeneChip Humane Genome U133 Plus 2.0 and NIH Taqman microRNA Array v.2 [5]) were retrieved from the openly accessible platform ArrayExpress (EMBL-European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, UK).

Cell culture. The human hepatoma derived Huh-7 and colon carcinoma derived Caco-2 cell lines (American Type Culture Collection, Molsheim, France) were cultured in RPMI-1640 and Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA), respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Primary human hepatocytes (PHHs) obtained from three patients suffering from primary or secondary liver carcinoma (ethical approval by the Local Ethical committee of the University of Munich, Germany, and the Ethics Committee of the Canton of Zurich, Switzerland) were isolated from the cancer-adjacent normal tissue and cultured as described [17]. PHHs were kept in maintenance medium including ultraglutamine for 5 h before further procedures. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Transient transfection with miR-192. To investigate the effect of miR-192 on *NR1H4* mRNA and protein expression, Huh-7 and Caco-2 cells were seeded in 12-well plates (8×10^4

and 4×10^5 cells/ml, respectively). After 24 h, cells were transfected with 100 nM hsa-mirVANA® miRNA mimics or Anti-miR™ miRNA inhibitors (hsa-miR-192-3p/-5p and corresponding negative controls, Life Technologies) using Lipofectamine® RNAiMax diluted in Opti-MEM I (both purchased from Invitrogen) at a final concentration of 3 mM. Hsa-miR-1 and its known suppressive effect on twinfilin-1 (TWF-1) expression were used as a positive control. After 4 h of incubation, the transfection medium was replaced with fresh complete growth medium. At 24, 48 and 72 h after transfection, total mRNA and protein were isolated using Trizol® reagent (Life Technologies) and RIPA buffer [50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (all from Sigma-Aldrich), 1 mM EDTA (Pharmacia Biotech, Uppsala, Sweden), 0.1% SDS, and 10% glycerol (both from Sigma-Aldrich)], respectively. FXR mRNA and protein were quantified in three independently performed experiments.

To examine miR-192-dependent expression of FXR target genes, Huh-7 cells were treated with 50 μM chenodeoxycholic acid (CDCA) (Sigma-Aldrich) for 24 h. Data obtained were merged from four experiments, and the three most representative values were averaged. To prove that miR-192 mimics control the expression of FXR target genes through FXR regulation, 100 nM FXR small interfering RNA (siRNA) or corresponding negative control (Life Technologies) were transfected simultaneously with the miRNA mimics for 48 h.

Reverse transcription and quantitative real-time PCR. Extracted RNA (1.5 μg) was transcribed into cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. A total of 2 μl of 1:5 diluted cDNA was mixed with 8 μl RT-PCR Universal Fast Master Mix (Applied Biosystems) including specific primers (TaqMan® Gene Expression Assays *NR1H4, SLCO1B3*, *SLC51A*, and *SLC51B*; Life Technologies) and subjected to real-time PCR. β-actin

was used as an internal control. To measure miR expression, 10 ng of extracted RNA was reverse transcribed into cDNA using a TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems) and specific stem-loop reverse transcription primers (TaqMan® MicroRNA Assays hsa-miR-192-3p and hsa-miR-192-5p; Life Technologies). RT-PCR was performed using 0.67 µl cDNA and 9.3 µl RT-PCR Universal Fast Master Mix (Applied Biosystems) including primers. U6snRNA was used as an internal control. Mean miR expression in three passages of cells were analyzed per cell line. All measurements were performed in triplicate.

Western blot analysis. Protein samples (17–30 µg) were diluted 1:5 with loading buffer, denatured, and separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membranes and preincubated for 1 h in blocking buffer and then for 16 h with the primary antibodies (anti-FXR, Santa Cruz Biotechnology, Dallas, TX, USA; anti-β-actin, Abcam, Cambridge, UK) at 4°C. After four washing steps, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit, Thermo Scientific, Waltham, MA, USA) for 1 h at room temperature. Protein bands were visualized using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany). β-actin was used as a housekeeping gene.

Transient cotransfection with miR-192-3p (miR-192*) and *NR1H4* 3′UTR plasmid constructs, and luciferase reporter assays. To examine the *NR1H4* 3′UTR as a target of miR-192-3p *in vitro*, luciferase assays were performed using a pmiRGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Fitchburg, WI, USA), containing both the coding sequences of firefly luciferase and Renilla luciferase (internal control). The *NR1H4* 3′UTR (accession number: NG_029843.1) was cloned into the pmiRGLO vector system using specific primers (Table 1). Plasmids were verified by Sanger sequencing. Huh-7 cells were

seeded in 48-well plates (1.6×10^5 cells/ml). After 24 h, the cells were transfected with 50 nM hsa-mirVANA® miRNA mimics or a negative control (hsa-miR-192-3p, Negative Control #1; Life Technologies) and 100 ng/well of plasmid DNA using Lipofectamine® 2000 (Invitrogen). The activities of firefly and Renilla luciferases were measured at 24 h after cotransfection using the Dual-Luciferase® Reporter 1000 Assay System (Promega) according to the manufacturer's protocol. Hsa-miR-21-5p and its effect on the miR-21 target sequence were used as a positive control. Analysis was performed using the GloMax® Multi Detection System (Promega). An empty PmiRGLO vector was used as a background control. QuikChange Multi Site-Directed Mutagenesis and QuikChange II XL Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA, USA) were used to introduce mutations into the miR-192-3p-binding sites using specific primers (Table 1). Three independent luciferase reporter assays were performed including wild-type and mutated target sequences.

Cell proliferation and invasion assays. Cell proliferation was determined by the alamarBlue® (ThermoScientific) cell viability reagent according to the manufacturer's instructions. Briefly, Huh-7 and Caco-2 cells were seeded in 96-well plates (7.5×10^4 and 2×10^5 cells/ml, respectively). After 24 h, cells were transfected with 100 nM miR-192 mimics, FXR siRNA or the corresponding negative controls. After 48, 72 and 96 h 10 µl of Alamar Blue was added to each well and cells were incubated for 4 h at 37°C. The absorbance was determined at 560/600nm using GloMax® Multi Detection System.

Cell invasion was determined with extracellular matrix-coated invasion chambers (QCM™ 24-well cell invasion assay, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Huh-7 and Caco-2 cells were harvested and resuspended in serum-free medium after pretreatment for 48 h with miR-192 mimics or negative control (cell density for

transfection: $1x10^5$ and $4x10^5$ cells/ml, respectively). Then, $1x10^5$ cells were plated into the invasion chamber, whereas the bottom well of the chamber contained 500 ul of the corresponding medium supplemented with 10% FBS. After 48 h of incubation, the invaded cells on the underside of the membrane were detached, lysed and stained with CyQuant® GR Dye (Millipore). Fluorescence was measured using a 490/510-570 filter set and GloMax® Multi Detection System.

Statistical analysis. Paired one-sample t-tests were performed to compare the effects of miR-192 mimics, antagomirs, FXR siRNA and the corresponding negative controls on FXR mRNA/protein expression levels and on cell proliferation/invasion. Luciferase activities were compared between miR-192-3p- and mock-transfected cells by one-way analysis of variance. All data obtained from transfection experiments were compared with negative control (NC) mimics or inhibitors, in which expression levels in mock-transfected cells were defined as 1 (except for cell proliferation assays, where values were normalized to the 48h time point of a particular condition). In general, the negative controls were not expected to bind to the *NR1H4* 3′UTR or to block the activity of endogenously expressed miR-192. Only experiments with verified positive controls were included in statistical analyses. The association between FXR and miR-192 expression levels was investigated in 27 colonic adenocarcinoma tissue samples by linear regression analyses excluding subjects that had undergone chemotherapy (E-GEOD-29623 [5]). Values are shown as averages ± standard deviation. A p value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using R-Software (version 2.15.2) and Graph Pad Prism (version 5.04).

RESULTS

The mature forms of pre-miR-192 derived from the 5′ and 3′ strands of the precursor are detectable in Huh-7 and Caco-2 cells. The online database miRBase lists two mature sequences for the human miRNA precursor pre-miR-192, miR-192-5p, and miR-192-3p. Quantitative measurement of the endogenous expression levels showed the presence of both strands at considerable amounts in Huh-7, PHH and Caco-2 cells. MiR-#-3p was expressed 10-fold less in Huh-7 cells, 32-fold less in Caco-2 cells, and 39-fold less in PHH cells compared with miR-#-5p. Furthermore, miR-192-3p showed a 9 to 10-fold higher expression in Caco-2 cells than in the hepatoma cell line or the primary human hepatocytes (data not shown).

Confirmation of the *NR1H4* 3′UTR as a target of miR-192-3p by luciferase reporter assays. The software tools miRANDA and DIANA-microT-CDS predicted binding of miR-192-3p to *NR1H4* transcript positions 199–227 and 324–352 (position relative to translational stop codon, NG_029843.1, Fig. 1A). No binding site within the *NR1H4* 3′UTR was predicted for miR-192-5p. Cotransfection of miR-192-3p mimics and the *NR1H4* 3′UTR target sequence into Huh-7 cells resulted in a 30% decrease in luciferase activity compared with the empty vector control ($p<0.01$). In contrast, luciferase activity remained unaffected by cotransfection of the *NR1H4* 3′UTR target sequence carrying the miR-192 binding sites in the mutated form compared with the wild-type construct (Fig. 1B), indicating a negative interaction of miR-192-3p with the predicted binding sites in the *NR1H4* 3′UTR.

MiR-192 attenuates endogenous *NR1H4* mRNA levels in Huh-7 and Caco-2 cells. To investigate the effect of miR-192 on endogenous FXR expression levels, Caco-2 and Huh-7 cells were transfected with 100 nM miR-192-3p or -5p mimics for 24 or 48 h. As shown in Fig. 2A, *NR1H4* mRNA levels were repressed to 75% and 65% by miR-192-3p and -5p mimics, respectively, in Huh-7 cells ($p<0.05$). In Caco-2 cells, a decrease by 15% and 28% in *NR1H4* mRNA expression was detected after transfection with miR-192-3p and -5p mimics for 24 h ($p<0.05$). To examine whether transfection of an antagomir could reverse the endogenous miR-192-dependent inhibitory effect on FXR expression, Caco-2 and Huh-7 cells were transfected with 100 nM anti-miR-192 inhibitors for 24 or 48 h, respectively. As shown in Fig. 2B, *NR1H4* mRNA levels were increased significantly by anti-miR-192-3p inhibitors in Caco-2 cells as well as anti-miR-192-5p inhibitors in Huh-7 cells compared with the anti-miR miRNA inhibitor negative control ($p<0.05$). Thus, the miR-192-dependent effect on FXR regulation appeared to be stronger in Huh-7 cells compared with Caco-2 cells.

MiR-192 suppresses FXR protein translation in Huh-7 and Caco-2 cells. Consistent with the miRNA-dependent effects on *NR1H4* mRNA expression, a 63% decrease in FXR protein expression was seen after transfection with miR-192-3p mimic in Huh-7 cells ($p<0.05$). Weaker downregulation by 14% was observed after transfection of miR-192-5p. A decrease by 14% and 43% in FXR protein expression was observed in Caco-2 cells after transfection with miR-192-3p and -5p, respectively (Fig. 3A). As shown in Fig. 3B, the endogenous miR-192-dependent suppressive effect on FXR protein expression was reversed upon transfection with anti-miR-192-3p or -5p inhibitors. In Huh-7 cells, an increase in FXR protein levels was not seen following transfection of the miR-192-3p antagomir, which could be explained by the lower expression of the 3′ in relation to the 5′ strand in Huh-7 compared to Caco-2 cells.

MiR-192 suppresses the expression of key FXR target genes. The bile acid transporters organic-anion transporting polypeptide 1B3 (OATP1B3, *SLCO1B3*) and organic solute transporters α/β (OST alpha/beta, *SLC51A/B*) were chosen as model genes to examine the effect of miR-192 on FXR-regulated expression of transport proteins. Huh-7 cells were transfected with 100 nM miR-192-3p or -5p for 48 h. 24 h after transfection, cells were treated with 50 µM CDCA for 24 h to activate FXR and compared with the miRNA mimic negative control. As shown in Fig. 4, S*LCO1B3*, *SLC51A* and *SLC51B* mRNA levels were decreased by either one or both strands of miR-192, suggesting a reduction of gene transcription secondary to reduced expression of the transcriptional activator FXR. This was confirmed by simultaneous FXR knockdown and miRNA transfection experiment (data not shown).

Linear regression analyses reveal an inverse association between the expression levels of miR-192 and FXR in colonic adenocarcinoma. To investigate whether the *in vitro* effects of miR-192 on FXR expression were reproducible *in vivo*, we analyzed 27 tissue samples from chemotherapy-untreated patients with primary colonic adenocarcinoma (Table 2). As shown in Table 3~~2~~ and Fig. 5, a significant inverse association in expression was observed for the *NR1H4* mRNA transcript coding for FXRα2(+) and hsa-miR-192-3p. FXRα2 isomers represent the most abundantly expressed FXR protein forms in colonic tissue, whereas the isomer FXRα1 is predominantly expressed in liver [30]. No significant associations were found for *NR1H4* mRNA and hsa-miR-192-5p expression, showing that the 5' strand does not confer pronounced *NR1H4* mRNA degradation *in vivo*. These findings support the regulatory effects of miR-192 on FXR expression in hepatoma and colon cancer-derived cell lines observed *in vitro*.

MiR-192 exhibits suppressive effects on proliferation of Huh-7 and Caco-2 cells. To investigate the functional significance of the observed miR-192/FXR interaction, we performed cell proliferation assays using Alamar Blue. As shown in Fig. 6, transfection with miR-192-3p mimic significantly reduces proliferation of Huh-7 and Caco-2 cells, whereby a stronger effect could be observed in the hepatoma cell line. These findings confirm the previously described suppressive effects of miR-192 on proliferation of the colon cancer cell lines HT-29, RKO and HCT116 [27]. Knockdown of FXR expression causes similar anti-proliferative effects in Huh-7 cells. In contrast, reduction in FXR expression does not seem to have any influence on the proliferative potential of Caco-2 cells. By performing cell invasion assays, we saw a trend towards a suppressive effect for both strands of the miR-192 precursor molecule on cell invasion of Huh-7 cells, however the results were not significant (data not shown). In a previous work, Lian et al. showed that miR-192 could significantly downregulate cell invasion of Huh-7 cells [19].

DISCUSSION

The aim of this study was to investigate whether FXR is a target of miR-dependent, post-transcriptional gene regulation. Regulation of FXR expression by miR-421 as an oncogenic miR in biliary tract cancer has already been postulated [36]. Our results show an additional, as yet uncharacterized role of miR-192 in regulating FXR expression.

Our *in vitro* experiments elucidated the inhibitory effect of miR-192 on FXR expression by transfecting colon and liver cell lines with miR-192 mimics and inhibitors. In the case of the 3′ strand, we found a significant miR-192-dependent inhibitory effect on *NR1H4*/FXR mRNA and protein expression in Huh-7 cells, whereas in Caco-2 cells only transfection with the anti-miR-192-3p inhibitor showed a relevant effect. A possible explanation for the observed weaker inhibitory effect of the miR-192-3p mimic in Caco-2 cells, compared with that in Huh-7 cells, may be the more abundant expression of miR-192 in the colon cell line. The observed miR-192*-dependent effects on *NR1H4* mRNA levels in both cell lines and our *in vivo* findings that showed a strong inverse miR-192* and FXRα2(+) correlation in colonic adenocarcinoma support our hypothesis of a strong endogenous effect of miR-192 on FXR expression. Degradation of *NR1H4* mRNA transcripts can be explained by perfect complementary interference of the miR-192-3p seed sequence at the two *in silico*-predicted binding positions of the *NR1H4*-3′UTR. The 5′ strand of miR-192 also appeared to repress FXR gene expression *in vitro*, albeit through a different mechanism. Transfections with the corresponding antagomirs support our findings with both mimics in our model cell lines.

According to the miRBase database, about 80 different human miRNA precursors can yield two abundant mature miRNAs, i.e. the 5′ strand (miR-#-5p) and 3′ strand (miR-#-3p) with different seed sequences and mRNAs as binding targets. There is increasing evidence for interplay between the 5′ and 3′ strands of the same precursor molecule targeting the same

group of genes, thereby reinforcing a certain phenotype [10], which can be supported by our data.

Hsa-miR-192 has been shown to play a crucial role in the pathogenesis of colon carcinoma, the third most common cancer in Western countries [6]. Because of its cancer stage-dependent decline in expression, miR-192 has been suggested to be a potential biomarker to predict metastasis in colon carcinoma patients [9]. Our findings regarding the inverse association of FXR and miR-192* expression in colonic cancer patients suggest that this miRNA-dependent mechanism of FXR regulation could play an important role in carcinogenesis. We additionally showed that miR-192 can suppress expression of the bile acid and anticancer drug transporter OATP1B3 and the bile acid transporter OSTα/β in a FXR-dependent manner.

It remains unclear whether restoration of miR-192 expression and the consequently diminished expression of FXR target genes would be beneficial for cancer patients, especially considering that miR-192 has been shown to possibly act as an oncogenic miRNA by downregulation of Smad interacting protein 1 in other inflammation-related cancers [21,26,35]. Therefore, it can be speculated that diminished expression of the transport protein OSTα/OSTβ, a heterodimer-forming bile acid transporter important for the excretion of bile acids from hepatocytes and enterocytes, may be a mechanism of intracellular bile acid accumulation promoting inflammation and/or cancer development in certain cases.

By performing cell proliferation and invasion assays, we were able to confirm the previously described suppressive role of miR-192 in liver and colon cancer progression. We observed a miR-192-associated anti-proliferative effect in Huh-7 cells that is stronger than in Caco-2 cells, where no noteworthy effects of the 3' strand arm could be seen on FXR protein levels. Furthermore, knockdown of FXR did not show any influence on Caco-2 cell proliferation. These observations support the hypothesis that the anti-proliferative effect of mR-192-3p may

be to some extent FXR-dependent. Future studies have to elucidate to what extent the miR-192/FXR interplay supports or inhibits tumor pathogenesis. Our results are in line with reports showing that enhanced FXR expression and the associated altered expression of FXR-regulated drug-uptake transporters is related to chemoresistance in cancer patients. Treatment of the colorectal adenocarcinoma cell line LS174T with cisplatin leads to a cisplatin-resistant phenotype that is accompanied by a 350-fold increase in FXR expression [22]. Furthermore, increased expression of OATP1B3 has been suggested to confer anti-apoptotic resistance to paclitaxel by altering p53-dependent pathways [18].

It may be of value to confirm the detected association between miR-192 and FXR expression in larger cohorts. We cannot exclude the contribution of other miRs or epigenetic factors to FXR regulation. However, a further step would be to systematically test all *in silico*-predicted miRs for effects on the *NR1H4*-3′UTR.

In conclusion, miR-192-5p and -3p negatively regulate the expression of FXR in a synergistic manner, thereby significantly decreasing the expression of FXR target genes OSTα/β and OATP1B3. We show a new miR-dependent mechanism of FXR regulation, which could affect the expression of FXR target genes and plays a role in the pathogenesis of liver and colon cancers and their response to anticancer therapies.

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DISCLOSURES

The authors have no disclosures to report.

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FIGURE LEGENDS

Fig. 1. (A) Predicted binding sites for the seed sequence of miR-192-3p in the 3′UTR of *NR1H4* mRNA using the bioinformatic tool miRANDA (www.microrna.org). (B) Relative reporter gene activities at 24 h after cotransfection of Huh-7 cells with the wild-type *NR1H4*-3′UTR target clone or mutated *NR1H4*-3′UTR target clone. The miR target clone control vector was used for normalization. Experiments were performed as triplicates and repeated three times. ** $p < 0.01$, ns = not significant.

Fig. 2. Effect of miR-192-5p and -3p mimics (A) and inhibitors (B) on *NR1H4* mRNA expression in Huh-7 and Caco-2 cells at 48 h and 24 h, respectively, after transfection. *NR1H4* mRNA expression relative to β-actin was determined by real-time PCR. The miR mimic/inhibitor negative control (NC) was used for normalization. Experiments were repeated three times. * $p < 0.05$, ns = not significant.

Fig. 3. Effect of miR-192-5p and -3p mimics on FXR protein expression in Huh-7 cells at 72 h after transfection and in Caco-2 cells at 48 h after transfection (A). Anti-miRNA-dependent effect on FXR protein levels (B). Caco-2 and Huh-7 cells were treated with anti-miR-192-5p and -3p inhibitors for 48 and 72 h. FXR protein expression relative to β-actin was determined by western blotting and densitometry. Bands of one representative blot are shown for each condition and cell line. Experiments were repeated three times. ** $p < 0.01$, * $p < 0.05$, ns = not significant.

Fig. 4. Effect of miR-192-5p and -3p mimics on mRNA expression of key FXR target genes important for bile acid homeostasis in Huh-7 cells at 48 h after transfection. *SLCO1B3*, *SLC51A*, and *SLC51B* mRNA expression relative to β-actin was determined by real-time PCR. The miR mimic negative control (NC) was used for normalization. Experiments were repeated four times. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant.

Fig. 5. Correlation between FXR and miR-192 expression in colonic adenocarcinoma-derived tissue samples from 27 patients by linear regression analysis. A significant inverse association was detected for miR-192-3p and the *NR1H4* mRNA transcript coding for FXRα2(+) (coefficient = -1.57/ $p = 0.0259$).

Fig. 6. Impact of miR-192-5p and -3p (A/C) or FXR siRNA (B/D) on proliferation of Huh-7 and Caco-2 cells using Alamar Blue assay. 48h values of each condition were used for normalization. Experiments were repeated four times. *** $p < 0.001$, ** $p < 0.01$,* $p < 0.05$.

TABLE LEGENDS

Table 1. Primer sequences used for subcloning and mutagenesis. Recognition sites for the restriction enzymes are underlined in the primers used for subcloning. *In silico*-predicted binding sites for hsa-miR-192-3p are underlined in the mutagenesis primers, whereas mutations inserted into these sites are indicated in bold. FXRwt_miR192* primers were used to optimize the second binding site for miR-192-3p.

Table 2. Clinical characteristics of the 27 patients showing primary colonic adenocarcinoma who were included into linear regression analysis.

Table 3~~2~~. Linear regression analysis of hsa-miR-192-3p with the *NR1H4* mRNA transcripts coding for FXRα2(+) and FXRα1(-), and miR-192-5p expression in primary colonic adenocarcinoma tissue obtained from 27 patients. miR-192-3p inversely correlates with FXRα2(+) expression.

~~Table 3. Clinical characteristics of the 27 patients showing primary colonic adenocarcinoma who were included into linear regression analysis.~~

Table 1: Primer sequences used for subcloning and mutagenesis

Oligonucleotide	Sequence (5' – 3')	Purpose
FXR_3UTR_FW	aggagctctggggattacaggggagg	Subcloning of *NR1H4* 3'prime end into pmirGlo
FXR_3UTR_RV	aggtcgacgccaagattgaatacaactct	Subcloning of *NR1H4* 3'prime end into pmirGlo
FXRmut_miR192*_FW1	ggaatcctgcattcta**gtcgccg**agccctgtttgcctaattaaattg	Multi-site directed mutagenesis
FXRmut_miR192*_FW2	gagttgtattcaatct**ggccg**tcgacctaatcccgcggc	Multi-site directed mutagenesis
FXRwt_miR192*_FW	gagttgtattcaatctt**g**gc**a**gtcgacctaatcccgcggc	Site-directed mutagenesis
FXRwt_miR192*_RV	gccgcgggattaggtcgac**t**gccaagattgaatacaactc	Site-directed mutagenesis

~~**Table 2: Linear regression analysis of miR-192-3p expression with FXRα2(+), FXRα1(-), and miR-192-5p expression in primary colonic adenocarcinoma**~~

~~Transcript~~	~~Coefficient~~	~~Std. Error~~	~~P-Value~~
~~FXRα2(+)~~	~~-1.57~~	~~0.66~~	~~2.59E-02~~
~~FXRα1(-)~~	~~1.87~~	~~0.70~~	~~1.39E-02~~
~~hsa-miR-192-5p~~	~~0.31~~	~~0.14~~	~~4.13E-02~~

~~Adjusted R-squared: 0.348~~

Table 2~~3~~: Clinical characteristics of 27 patients with primary colonic adenocarcinoma

Parameters	Category	N(%)
Gender	male	15(55.6)
	female	12(44.4)
AJCC stage	pT1	6(22.2)
	pT2	13(48.2)
	pT3	4(14.8)
	pT4	4(14.8)
Tumor grade	1	3(11.1)
	2	20(74.1)
	3	4(14.8)
Treatments	Chemotherapy-naive	27(100)

Table 3: Linear regression analysis of miR-192-3p expression with FXRα2(+), FXRα1(-), and miR-192-5p expression in primary colonic adenocarcinoma

Transcript	Coefficient	Std. Error	P-Value
FXRα2(+)	-1.57	0.66	2.59E-02
FXRα1(-)	1.87	0.70	1.39E-02
hsa-miR-192-5p	0.31	0.14	4.13E-02

Adjusted R-squared: 0.348